INFLAMMATORY BOWEL DISEASE AND IRRITABLE BOWEL SYNDROME IBD-FIRST CHEK DIAGNOSTIC PANEL

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application No. 60/421,395, filed October 25, 2002.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[0002] Not applicable.

TECHNICAL FIELD

[0003] A method for the differentiation of inflammatory bowel disease (IBD) from irritable bowel disease (IBS) followed by distinguishing ulcerative colitis from Crohn's disease and other gastrointestinal illnesses. This highly differential method first uses the presence of elevated fecal lactoferrin as a marker of intestinal inflammation to differentiate IBD from IBS. Patients suspected of IBD are then analyzed for anti-*Saccharomyces cerevisiae* antibodies (ASCA) as an indicator of Crohn's disease and fecal anti-neutrophil cytoplasmic antibodies (ANCA) as an indicator of ulcerative colitis. IBD patients are further monitored for intestinal inflammation using lactoferrin to evaluate the effectiveness of medical therapy and predict relapse. The apparatus comprises either a qualitative enzyme-linked immunoassay or other immunoassay that utilizes antibodies for the measurement of total endogenous lactoferrin, ASCA and ANCA in human feces. The method and apparatus can be used by healthcare providers to identify IBD and distinguish ulcerative colitis from Crohn's disease.

BACKGROUND OF THE INVENTION

[0004] An estimated 1 million Americans suffer from chronic inflammatory bowel disease (IBD) and 20 million Americans suffer from irritable bowel syndrome (IBS). IBD, comprised of both Crohn's Disease (CD) and ulcerative colitis (UC), is characterized by a chronic inflammatory response that results in histologic damage to the intestinal lining. Both CD and UC exhibit large numbers of leukocytes that migrate to the mucosa and into the intestinal lumen. Both diseases oscillate between active (i.e., presence of intestinal inflammation) and inactive (i.e., minimal to no intestinal inflammation) stages of disease activity. Active IBD can include symptoms such as bloody diarrhea, abdominal pain, and fever. The inactive stage has minimal to no intestinal inflammation and lacks severe gastrointestinal illness.

[0005] Patients who have active IBD but who exhibit mild signs and symptoms may be difficult to distinguish from patients with active IBS, an intestinal disorder of motility and the intestinal nervous system. Unlike IBD, IBS does not involve intestinal inflammation. In persons with IBS, the intestine appears normal upon endoscopic examination and leukocytes are not present in the mucosa or in fecal specimens. Symptoms can mimic those of IBD and include bloating, diarrhea, constipation, and severe and often debilitating abdominal pain. It is estimated that at least 20 million Americans suffer from IBS.

[0006] The similarity in symptoms between IBS and IBD renders rapid diagnosis difficult. However, given the potential severity of untreated IBD, differential diagnosis is crucial. The diagnosis of gastrointestinal illnesses, in general, is aided by diagnostic tests such as enzyme-linked immunosorbant assays (ELISAs), latex agglutination and lateral

flow immunoassay. These tests are rapid and inexpensive methods for detecting markers in feces for enteric pathogens and inflammation. One marker of particular interest that has been found to be most specific for leukocytes in fecal specimens is lactoferrin. Human lactoferrin is an 80 kilodalton glycoprotein. This iron-binding protein is secreted by most mucosal membranes. It is a major component of the secondary granules found in polymorphonuclear neutrophils (PMNs), a primary component of the acute inflammatory response. Other hematopoietic cells such as monocytes and lymphocytes, do not contain lactoferrin, whereas various bodily secretions contain levels in the mg/mL range. During the process of inflammation, PMNs infiltrate the mucosa lining of the small and large intestine. This increase in the number of activated tissue leukocytes and exudation of plasma from ulcerated mucosa results in an increase in the level of lactoferrin found in feces. The protein is resistant to proteolysis and, as such, it provides a useful non-invasive fecal marker of intestinal inflammation.

[0007] Human lactoferrin has been used as a marker for fecal leukocytes in a number of applications. For instance, fecal lactoferrin has been used as a marker for leukocytes to distinguish noninflammatory diarrhea from inflammatory diarrhea, as disclosed in U.S. Patent No. 5,124,252. Noninflammatory diarrhea caused by agents such as rotavirus, Norwalk-like agents and cholera, typically causes minimal to no intestinal damage and patients respond readily to oral rehydration. Inflammatory diarrheas include those caused by enteric pathogens such as *Clostridium difficile*, *Shigella* species, *Salmonella* species, *Campylobacter jejuni* and *Entamoeba histolytica* and those that have no clearly defined infectious agent such as CD and UC. U.S. Patent No. 5,124,252 discloses an *in vitro* test for fecal leukocytes that aids in distinguishing

inflammatory from noninflammatory diarrhea. The '252 patent discloses testing fecal samples suspected of containing leukocytes with an assay that utilizes an antibody for lactoferrin to determine the presence of leukocytes in the fecal sample.

[0008] Human lactoferrin also has been used as a marker for diagnosis of inflammatory gastrointestinal disorders, colon polyp and colorectal cancer as disclosed in U.S. Patent No. 5,552,292. However, neither the method of the '252 patent nor that of the '292 patent disclose utility in distinguishing IBS from IBD. The samples tested by the assay of the '252 patent are samples suspected of containing leukocytes. This suspicion is owed to the patient presenting with diarrhea. However, 25-50% of persons having IBD do not present with diarrhea and, thus, the '252 patent does not relate to diagnosing etiology in such patients. As for the '292 patent, the disclosed method utilizes a 1:100 sample dilution which does not allow for accurate quantitation of lactoferrin levels. Further, the '292 patent discloses using partial forms of molecules for testing and not total endogenous lactoferrin, again affecting the accuracy of the quantitation. The method of the '292 patent also does not relate to utilizing lactoferrin levels to distinguish IBD from IBS. The population tested in the '292 patent, while including persons with UC and CD, did not include persons having IBS.

[0009] IBD is comprised of both Crohn's disease and ulcerative colitis. These two distinct diseases require a rapid differential diagnosis for optimal treatment. Crohn's disease may involve the entire gastrointestinal tract and include inflammation extending into the transmural mucosa, whereas ulcerative colitis affects solely the large bowel and includes inflammation of the innermost lining. Conventional methods to differentiate

between Crohn's disease and ulcerative colitis utilizing multiple endoscopy examinations and histological analysis may take years to confirm a diagnosis.

[0010] U.S. Patent No. 6,218,120 discloses a method of determining the presence of serum ANCA as a marker to diagnose IBD. However, it does not disclose a method for diagnosing ulcerative colitis in a patient diagnosed with IBD.

[0011] Serological methods for the differential diagnosis of CD and UC also are known in the art. For example, it is known to use the presence of serum anti-*Saccharomyces cerevisiae* antibodies (ASCA) to diagnose CD. See Main et al., Antibody to *Saccharomyces cervisiae* (baker's yeast) in Crohn's disease, BMJ Vol. 297 (October 29, 1988); Broker et al., A Murine Monoclonal Antibody Directed Against a Yeast Cell Wall Glycoprotein Antigen of the Yeast Genus *Saccharomyces*, FEMS Microbiology Letters 118 (1994), 297-304. It is further known in the art to use the presence of serum ASCA to diagnose clinical subtypes of UC and CD in patients presenting with established diagnoses. For example, U.S. Patent No. 5,968,741 discloses utilizing the presence of serum ASCA to diagnose a medically resistant clinical subtype of UC in patients presenting with an established diagnosis of UC. Similarly, U.S. Patent No. 5,932,429 discloses utilizing the presence of serum ASCA to diagnose a clinical subtype of CD in patients presenting with an established diagnosis of CD.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

[0012] FIG. 1 is a graphical representation of a standard curve of purified anti-*Saccharomyces cerevisiae* antibodies in accordance with an embodiment of the present invention; and

[0013] FIG. 2 is graphical representation of a standard curve of anti-neutrophil cytoplasmic antidodies in accordance with an embodiment of the present invention.

DETAILED DESCRIPTION OF THE INVENTION

[0014] The present invention relates a lactoferrin immunoassay used to determine the presence of elevated lactoferrin as an indicator of intestinal inflammation thus aiding in the differentiation of IBD from IBS, and ANCA and ASCA immunoassays to differentiate between ulcerative colitis and Crohn's disease. The test results may be used to determine appropriate treatment for ulcerative colitis and Crohn's disease patients. Qualitative immunoassays such as enzyme–linked immunoassays and lateral flow dipsticks that utilize monoclonal and polyclonal antibodies to human ANCA and ASCA may be used distinguish between ulcerative colitis and Crohn's disease. Bodily secretions, as used herein may include, but are not limited to, feces and mucosal secretions, whole blood, serum, plasma, saliva or other bodily fluid or tissue.

[0015] In the qualitative assay, the bodily secretions are diluted and added to a well containing the immobilized antibodies to lactoferrin or antigens of *Saccharomyces cerevisiae* or neutrophil cytoplasmic antigens. If endogenous lactoferrin or ASCA or ANCA is present, it will bind to the well containing immobilized antibodies or antigens during an incubation step. Following the incubation, antibodies to human lactoferrin or polyvalent antibodies to human immunoglobulin coupled to horseradish peroxidase enzyme (conjugate) is added and allowed to bind to captured lactoferrin or ANCA or ASCA. Unbound conjugate is washed from the well and one component substrate (tetra-methyl-benzidene and hydrogen peroxide) is added for color development. Following the

substrate incubation, the reaction is stopped by acidification and the optical density (OD) is determined spectrophotometrically at 450 nm.

[0016] The particular embodiments described herein are intended in all respects to be illustrative rather than restrictive. Alternative embodiments will become apparent to those skilled in the art to which the present invention pertains without departing from its scope.

Example 1
Lactoferrin Qualitative Assay

a. Establishment of Optimal Sample Dilution Factor and Optical Density

[0017] The assay of the present invention was designed and developed to detect levels of fecal lactoferrin at a lower level detectable by predicate devices, specifically the LEUKO-TEST®. The lower limit of detection of the LEUKO-TEST® is 256 ng/mL with purified human lactoferrin. In the LEUKO-TEST®, a specimen dilution of 1:50 and a minimum limit of detection of 256 ng/mL provides a lower limit of detection in fecal specimens of approximately 12 µg/mL. A specimen dilution of 1:400 and a minimum detection limit for the assay of the present invention of 32 ng/mL also provides a lower limit of detection in fecal specimens of approximately 12 µg/mL. Accordingly, a 1:400 specimen dilution was chosen for the assay of the present invention. Similarly, an optical density of 0.200 OD_{450} for the assay was chosen. (As used herein, OD_{450} indicates an optical density obtained spectrophotometrically at 450 nm on a single wavelength spectrophotometer.)

[0018] It will be understood and appreciated by those of skill in the art that the preferred dilution factor and optical densities have been determined based upon reagents currently available and deemed to be optimal. However, reagents other than those now

desired may become improved and desirable over time. Variations in reagents may produce preferable/optimal dilution factors and/or optical densities other than those determined herein. Such variations are contemplated to be within the scope of the present invention. The key to determining optimal values is based upon sensitivity as more fully described below.

[0019] To verify that the 1:400 specimen dilution provides the most desirable sensitivity with the current reagents, 121 fecal specimens were analyzed comparing a 1:400 dilution to a 1:800 dilution. (Sensitivity is calculated herein by dividing the number of samples taken from subjects with IBD which produce a positive result in the assay by the number of samples taken from subjects with IBD.) Test results additionally were evaluated comparing OD_{450} values of 0.200 to OD_{450} values of 0.300. Results were compared with microscopy for fecal leukocytes and with the LEUKO-TEST®. The results are summarized in Tables 1-8 below.

Table 1: Comparison of the ELISA with microscopy for fecal leukocytes using a 1:400 dilution and an OD_{450} of 0.200

ELISA vs. Microscopy (N=121)	Microscopy positive	Microscopy negative
ELISA positive	32	42
ELISA negative	2	45

Relative Sensitivity	94.0%
Relative Specificity	52.0%
Correlation	64.0%

Table 2: Comparison of the ELISA with microscopy for fecal leukocytes using a 1:400 dilution and an OD_{450} of 0.300

ELISA vs. microscopy (N=121)	Microscopy positive	Microscopy negative
ELISA positive	31	31
ELISA negative	3	56

Relative Sensitivity	91.0%
Relative Specificity	64.0%
Correlation	72.0%

Table 3: Comparison of the ELISA with microscopy for fecal leukocytes using a 1:800 dilution and an OD_{450} of 0.200

ELISA vs. microscopy (N=121)	Microscopy positive	Microscopy negative
ELISA positive	30	31
ELISA negative	4	56

Relative Sensitivity	88.0%
Relative Specificity	64.0%
Correlation	77.0%

Table 4: Comparison of the ELISA with microscopy for fecal leukocytes using a 1:800 dilution and an OD_{450} of 0.300

ELISA vs. microscopy (N=121)	Microscopy positive	Microscopy negative
ELISA positive	26	24
ELISA negative	8	63

Relative Sensitivity	77.0%
Relative Specificity	72.0%
Correlation	74.0%

Table 5: Comparison of the ELISA with the LEUKO-TEST® using a 1:400 dilution and an OD_{450} of 0.200

ELISA vs. LEUKO-TEST® (N=121)	LEUKO-TEST® Positive	LEUKO-TEST® negative
ELISA positive	43	31
ELISA negative	5	42

Relative Sensitivity	89.6%
Relative Specificity	57.5%
Correlation	70.2%

Table 6: Comparison of the ELISA with the LEUKO-TEST® using a 1:400 dilution and an OD_{450} of 0.300

ELISA vs. LEUKO-TEST® (N=121)	LEUKO-TEST® Positive	LEUKO-TEST® negative
ELISA positive	41	21
ELISA negative	7	52

Relative Sensitivity	85.0%
Relative Specificity	71.2%
Correlation	77.0%

Table 7: Comparison of the ELISA with the LEUKO-TEST® using a 1:800 dilution and an OD_{450} of 0.200

ELISA vs. LEUKO-TEST® (N=121)	LEUKO-TEST® Positive	LEUKO-TEST® negative
ELISA positive	39	22
ELISA negative	9	51

Relative Sensitivity	81.3%
Relative Specificity	69.9%
Correlation	74.4%

Table 8: Comparison of the ELISA with the LEUKO-TEST® using a 1:800 dilution and an OD_{450} of 0.300

ELISA vs. LEUKO-TEST® (N=121)	LEUKO-TEST® Positive	LEUKO-TEST® negative
ELISA positive	34	16
ELISA negative	14	57

Relative Sensitivity	70.8%
Relative Specificity	78.1%
Correlation	75.2%

[0020] In summary, a fecal specimen dilution of 1:400 and an assay OD_{450} of 0.200 showed the highest level of sensitivity with the current reagents. Accordingly, these conditions were determined to be optimal for the assay of the present invention. Normal fecal specimens contain low levels of lactoferrin and the 1:400 dilutions have been determined to be optimal in detecting an increase in lactoferrin over background levels. The use of dilutions lower than 1:400 may result in positive test results due to the presence of normal lactoferrin levels.

b. Collection of Specimens and Preparation of Dilutions

[0021] Standard collection and handling procedures typically used for fecal specimens for culture may be used in collecting samples for the assay of the present invention. In the preferred embodiment, fecal specimens are to be tested within twenty-four hours of collection. However, if the assay is not to be performed within forty-eight hours of collection, it is preferred that the specimens be stored at -20°C or lower. Additionally, it is preferred that collected specimens be transported and diluted in the Diluent as soon as possible after collection and, once diluted, that the specimens be stored at between about 2°C and about 8°C. It is preferred that the specimens be mixed (i.e., using a vortex mixer) thoroughly prior to performing the assay of the present invention. This includes complete mixing of the specimen prior to transfer to the Diluent, as more fully described below, as well as complete mixing of the diluted specimen prior to performing the assay.

[0022] The following method was used to prepare a diluted specimen from a liquid fecal specimen. Two plastic tubes were set up for each specimen to be tested. For each specimen, 950µL of 1X Diluent (prepared as more fully described below) subsequently was added to each of the two tubes. Using a transfer pipette, one drop (i.e., approximately 50 µL) of liquid fecal specimen was added to one of the tubes and thoroughly mixed using a vortex mixer. Subsequently, one drop of the diluted specimen was transferred into the second tube containing 950 µL of 1X Diluent (prepared as more fully described below). The result was a 1:400 dilution of the specimen in the second tube. Thus, only the second tube was used for the remainder of the test procedure.

[0023] The following method was used to prepare a diluted specimen from a formed or solid fecal specimen. Two plastic tubes were set up for each specimen to be tested. For each specimen, 1.9 mL of 1X Diluent (prepared as more fully described below) was added to only one of the two tubes. Subsequently, 0.10 g of fecal specimen were added to this tube (1:10) and thoroughly mixed using a vortex mixer. Next, 950 µL of the 1X Diluent (prepared as more fully described below) was added to the second tube and one drop (i.e., approximately 50 µL) of the previously diluted specimen is transferred into the second tube. The result was a 1:400 dilution of the specimen in the second tube. Thus, only the second tube was used for the remainder of the test procedure.

[0024] The specimen in the second tube prepared according to either of the above procedures was mixed in a vortex mixer for approximately ten seconds and subsequently stored at between about 2°C and about 8°C until the remainder of the test procedure was performed. Prior to transferring the diluted specimen into a microtiter well according to the test procedure, as more fully described below, the specimen was thoroughly mixed in the vortex mixer once again. This procedure sought to ensure thorough mixing of the specimen.

c. Necessary Test Reagents and Preparation Thereof

[0025] A number of reagents were necessary to carry out the preferred embodiment of the qualitative assay of the present invention. These reagents included 10X Diluent, 1X Diluent, Conjugate, Substrate, Positive Control, Wash Buffer Solution and Stop Solution. The 10X Diluent was a 10X concentrate of buffered protein solution containing 0.2% thimerosal as a preservative. The Diluent was supplied as a 10X concentrate. Therefore, to prepare the 1X Diluent necessary for the assay of the present

invention, a total volume of 400 mL was diluted by adding 40 mL of the 10X concentrate to 360 mL of deionized water. Any unused 1X Diluent was stored at between about 2°C and about 8°C.

[0026] The Conjugate used with the assay of the present invention preferably comprises rabbit polyclonal antibody specific for human lactoferrin conjugated to horseradish peroxidase and in a buffered protein solution containing 0.02% thimerosal as a preservative. The Substrate used with the assay of the present invention preferably comprises a solution containing tetra-methyl-benzidine substrate and peroxidase. The Positive Control used with the assay of the present invention preferably comprises human lactoferrin in a buffered protein solution containing 0.02% thimerosal as a preservative. The Stop Solution used with the assay of the present invention preferably comprises 0.6 N sulfuric acid.

[0027] The Wash Buffer Solution used with the assay of the present invention was supplied as a 20X concentrate containing phosphate buffered saline, detergent and 0.2% thimerosal as a preservative. To prepare the 1X Wash Solution necessary for the assay of the present invention, a total volume of one liter of concentrate was diluted by adding 50 mL of the concentrate to 950 mL of deionized water. Any unused 1X Wash Solution was stored at between about 2°C and about 8°C.

[0028] Microassay plates containing twelve strips and eight wells per strip are preferred for the assay of the present invention. Each specimen and each control requires a single coated well. To prepare the plates, each strip was coated with purified polyclonal antibody specific for lactoferrin. Microassay plates were stored with desiccant.

[0029] All reagents were stored at room temperature prior to use in the assay of the present invention.

[0030] The present invention includes a kit designed and prepared for carrying out the quantitative assay. In the preferred embodiment, the kit contains 40 mL 10X Diluent, 7 mL Conjugate, 14 mL Substrate, 3.5 mL Positive Control, 50 mL Wash Buffer Solution, 7 mL Stop Solution and one microassay plate stored with desiccant. The assay of the present invention utilizes antibodies to human lactoferrin. The microassay plate supplied with the kit contains immobilized polyclonal antibody against lactoferrin. The detecting antibody consists of polyclonal antibody conjugated to horseradish peroxidase.

d. Test Procedure

[0031] To perform the qualitative assay of the present invention, initially the number of wells needed was determined. Each specimen or control required one well and, therefore, the number of wells was determined accordingly. Next, one drop (i.e., about 50 μL) of Positive Control was added to a single well designated the Positive Control Well and one drop (i.e., about 50 μL) of 1X Diluent was added to a single well designated the Negative Control Well. Subsequently, two drops (i.e., about 100 μL) of 1:400 diluted specimen (prepared according to the above procedure) was added to a third well and all wells were incubated at about 37°C (± 2°C) for approximately thirty minutes. After incubation, the contents of the assay wells was discarded into a discard pan.

[0032] Next, each well was washed using 1X Wash Solution (prepared as described above) and placed in a squirt bottle with a fine-tipped nozzle. In this manner, the 1X Wash Solution was directed into the bottom of each of the wells with some force.

Each well was filled with the 1X Wash Solution and the contents thereof subsequently discarded into a discard pan. The microassay plate was then inverted and slapped on a dry paper towel. This wash procedure was performed a minimum of four times using a dry paper towel each time. If any particulate matter was observed in the wells, the washing procedure was continued until all the matter was removed.

[0033] Subsequently, one drop (i.e., about 50μL) of Conjugate was added to each well and the wells were incubated at about 37°C (±2°C) for approximately thirty minutes. After incubation, the contents of the assay wells were discarded into a discard pan and the washing procedure was repeated. Next, two drops (i.e., about 100 μL) of Substrate were added to each well and the wells were gently tapped to mix the contents. The wells were then incubated at room temperature for approximately fifteen minutes. The wells were gently tapped a couple of times during the incubation period.

[0034] Next, one drop (i.e., 50 μL) of Stop Solution was added to each well and the wells were gently tapped. The wells were allowed to sit at room temperature for about two minutes before reading. The addition of Stop Solution converted the blue color to a yellow color which could then be quantified by measuring the optical density at 450 nm on a microplate ELISA reader. The instrument was blanked against the negative control and the underside of each well was wiped before measuring the optical density. Optical densities (OD_{450} and $OD_{450/620}$) were recorded for the Positive Control Well, the Negative Control Well and each specimen tested. ("$OD_{450/620}$" as used herein indicates an optical density obtained spectrophotometrically at 450/620 nm on a dual wavelength spectrophotometer.) Readings of duplicate wells were averaged before the results were interpreted.

[0035] The specified test procedure represents the preferred embodiment as optimal results are obtained by following the procedure specified because the reagents, concentrations, incubation conditions, and processing specifications have been optimized for sensitivity and specificity. Accordingly, alterations of the specified procedure and/or of the indicated test conditions may affect the sensitivity and specificity of the test.

e. Quality Control

[0036] The positive and negative control must meet certain criteria for the test to be valid. First of all, the Positive Control Well must be a visible yellow color and, when read on a spectrophotometer, it must have an OD_{450} and $OD_{450/620}>0.500$. The Negative Control Well must have an $OD_{450}<0.200$ or an $OD_{450/620}<0.160$. To ensure that carryover has not occurred, testing should be repeated if a sample gives a weak positive result (i.e, <0.400) and is adjacent to a strong positive well.

f. Interpretation of Results

[0037] Optical densities were measured at 450 nm on a single wavelength spectrophotometer and at 450/620 nm on a dual wavelength spectrophotometer. On a single wavelength spectrophotometer, an OD_{450} of less than 0.200 indicated a negative result and an OD_{450} of greater than or equal to 0.200 indicated a positive result. On a dual wavelength spectrophotometer, an $OD_{450/620}$ of less than 0.160 indicated a negative result and an $OD_{450/620}$ of greater than or equal to 0.160 indicated a positive result.

[0038] A positive test result indicated the specimen contained elevated levels of lactoferrin when compared with a reference value established for healthy control subjects. A negative test result indicated the specimen did not contain elevated levels of lactoferrin relative to samples from healthy control subjects.

g. Results

[0039] One hundred forty-nine subjects having IBD were tested according to the above procedure. Seventy-seven of the subjects, or 51.7%, were male and seventy-two of them, or 48.3%, were female. The tested male to female ratio closely approximates the 1:1 ratio observed in the general IBD patient population. Ages of the subjects ranged from 3 years to 78 years and thirty-two subjects, or 22%, were 16 years of age or younger. Seventy-seven subjects, or 51.7%, had CD and seventy-two of them, or 48.3% had UC.

[0040] Thirty-one subjects having IBS were tested. Six of the subjects, or 19.3%, were male and twenty-five of them, or 80.7%, were female. The tested male to female ratio closely approximates the 1:3 ratio observed in the general IBS population. Ages of the subjects ranged from 19 years to 78 years.

[0041] Fifty-six healthy subjects also were tested as controls. Twenty-eight of the subjects, or 50%, were male and twenty-eight of them, or 50%, were female. Ages of the subjects ranged from infants to 79 years. A summary of the tested subject population is illustrated in Table 9.

Table 9: Summary of Subject Population

Summary of Clinical Histories (N=180)	Total Subjects
Total number of IBD patients	149
No. Males	77
No. Females	72
Total number of patients with CD	77
No. Males	43
No. Females	34
Total number of patients with UC	72
No. Males	34
No. Females	38
Total number of patients with irritable bowel syndrome	31
No. Males	6
No. Females	25
Total number of healthy persons	56
No. Males	28
No. Females	28

[0042] Fecal specimens were collected from each enrolled subject and stored at -70°C until tested. Sample consistencies ranged from liquid to solid, numbers for which are illustrated in Table 10 for each subject group. As can be seen, forty-five of the IBD specimens were liquid specimens, sixty-two were semi-solid specimens, and forty-two were solid specimens. One of the IBS specimens was a liquid specimen, thirteen were semi-solid specimens, and seventeen were solid specimens. All of the specimens from healthy control subjects were solid.

Table 10: Summary of Specimen Consistencies for Each Subject Group

Summary of Stool Specimens (N=236)	Total Specimens
Total number of IBD patients (CD and UC)	**149**
Total number of liquid specimens	45
Total number of semi-solid specimens	62
Total number of solid specimens	42
Total number of patients with IBS	**31**
Total number of liquid specimens	1
Total number of semi-solid specimens	13
Total number of solid specimens	17
Total number of healthy persons	**56**
Total number of liquid specimens	0
Total number of semi-solid specimens	0
Total number of solid specimens	56

[0043] The level of fecal lactoferrin in each specimen was determined using the qualitative lactoferrin ELISA as previously described. A specimen dilution of 1:400 was used. Results were reported as positive if an optical density of greater than or equal to 0.200 was observed. Conversely, results were reported as negative if an optical density of less than 0.200 was observed.

[0044] Of the IBD subject group, ninety-two subjects had active disease and fifty-seven had inactive disease. Of the active group, a total of eighty subjects, or 87.0%, tested positive in the assay. Of the inactive group, a total of thirty-two subjects, or 56.1%, tested positive. Of the forty-one subjects having active UC, a total of thirty-six subjects, or 87.8% tested positive in the assay. Of the fifty-one subjects having active CD, forty-four, or 86.3%, tested positive. All thirty-one patients having active IBS and all fifty-six healthy control subjects tested negative in the assay. A summary of assay test

results is illustrated in Table 11 and various individual comparisons are illustrated in Tables 12, 13 and 14, as more fully described below.

Table 11: Summary of ELISA test Results for CD, UC, Active IBS, and Healthy Control Subjects

Clinical Assessments N = 236	Total	ELISA Positive	ELISA Negative
Total IBD	149	75.2% (112)	24.8% (37)
Active	92	87.0% (80)	13.0% (12)
Inactive	57	56.1% (32)	43.0% (25)
Total CD	77	77.9% (60)	22.1% (17)
Active	56	86.3% (44)	13.7% (7)
Inactive	26	61.5% (16)	38.5% (10)
Total UC	72	72.2% (52)	27.7% (20)
Active	41	87.8% (36)	12.2% (5)
Inactive	31	51.6% (16)	48.4% (15)
Total Active IBS	31	0	100.0% (31)
Total Healthy Persons	56	0	100.0% (56)

[0045] When distinguishing samples from active IBD subjects from subject samples having IBS or from healthy control samples, the ELISA exhibited a sensitivity of 87% and specificity of 100%. Sensitivity was calculated by dividing the number of persons having IBD and testing positive in the ELISA by the number of subjects having IBD. Specificity was calculated by dividing the number of subjects having IBD and testing positive in the ELISA by the number of subjects testing positive in the ELISA. The predictive positive and negative values were 100% and 87.9%, respectively, and the correlation was 93.3%. These results are summarized in Table 12.

Table 12: Statistical Evaluation using the ELISA to Distinguish Active IBD from IBS/Healthy Control Subjects

N=179	Active IBD	IBS/Healthy Controls
ELISA positive	80	0
ELISA negative	12	87

Sensitivity	87.0%
Specificity	100%
Predictive Positive Value	100%
Predictive Negative Value	87.9%
Correlation	93.3%

[0046] When distinguishing samples from active UC subjects from subject samples having IBS or from healthy control subjects, the ELISA exhibited a sensitivity of 87.8% and a specificity of 100%. The predictive positive and negative values were 100% and 94.6%, respectively, and the correlation was 96.1%. These results are summarized in Table 13.

Table 13: Statistical Evaluation using the ELISA to Distinguish Active UC from IBS/Healthy Control Subjects

N=128	Active UC	IBS/Healthy Controls
ELISA positive	36	0
ELISA negative	5	87

Sensitivity	87.8%
Specificity	100%
Predictive Positive Value	100%
Predictive Negative Value	94.6%
Correlation	96.1%

[0047] When distinguishing subject samples having active CD from subject samples having IBS or from healthy control samples, the ELISA exhibited a sensitivity of 86.3% and a specificity of 100%. The predictive positive and negative values were 100% and 92.6%, respectively, and the correlation was 94.9%. These results are summarized in Table 14.

Table 14: Statistical Evaluation using the ELISA to Distinguish Active CD from IBS/Healthy Control Subjects

N=138	Active UC	IBS/Healthy Controls
ELISA positive	44	0
ELISA negative	7	87

Sensitivity	86.3%
Specificity	100%
Predictive Positive Value	100%
Predictive Negative Value	92.6%
Correlation	94.9%

h. Reproducibility and Precision

[0048] The inter-assay variation was determined by analyzing eight lactoferrin-negative and eight lactoferrin-positive fecal specimens over a three day period. The average % Coefficient of Variation (CV) was 23.5% for the positive specimens and 7.4% for the negative specimens. The intra-assay variation was determined by analyzing twelve fecal specimens using six replicates in one lot of kits. The intra-assay analysis ranged in %CV from 2.7 to 24.0 with an average of 8.7%.

Example 2
Lactoferrin Quantitative Assay

[0049] In the quantitative assay of the present invention, fecal specimens preferably are serially diluted ten-fold and added to microtiter wells containing immobilized polyclonal antibodies against human lactoferrin. If endogenous lactoferrin is present, it will bind to the antibodies during an incubation at approximately 37°C. Following the incubation, conjugate comprised of polyclonal antibodies coupled to horseradish peroxidase enzyme is added and allowed to bind to captured lactoferrin. Unbound conjugate is then washed from the well and a component substrate (e.g., tetra-methyl-benzidene and hydrogen peroxide) is added for color development. Following the substrate incubation, 0.6N sulfuric acid is added to quench the reaction and the absorbance or optical density (OD) is obtained spectrophotometrically at 450 nm on a single wavelength device. Fecal lactoferrin concentrations are determined by comparison to a standard curve generated using purified human lactoferrin.

a. Preparation of Standard Curve

[0050] A 1 mg/mL stock solution of purified human lactoferrin, manufactured by Sigma Immunochemicals of St. Louis, Missouri, was prepared using 10 mg of lactoferrin dissolved in 10 mL of sterile phosphate buffered saline (PBS) at pH 7.4. Serial two-fold dilutions of lactoferrin were made using the range of approximately 6 to 100 ng/mL in Diluent. For the analysis, 0.1 mL of each standard was assayed in duplicate. Optical densities (OD_{450}) were determined and plotted versus lactoferrin concentration to generate standard curves. The linear portion of the curve was determined by linear regression analysis using the Log-Log method (Microsoft EXCEL, Microsoft R Office). The lowest dilution of specimen that gave an OD_{450} within the linear portion of the curve

was used to determine the lactoferrin concentration. The final concentration was obtained by multiplying the concentration by the dilution factor.

b. Quantitative Test Procedure

[0051] In order to assess the ability of the quantitative ELISA to measure the level of fecal lactoferrin, two fecal specimens collected six weeks apart from six female and five male adults were diluted and then spiked with lactoferrin to a concentration of 25 ng/mL. The estimated lactoferrin that was determined represents the level of lactoferrin determined from a standard curve generated with the quantitative ELISA. The % Variation represents the difference between the actual amount used to spike the sample and the estimated amount. Under these conditions, the variations ranged from 1.0% to 85.8% for females and 8.8% to 47.0% for males. Results showed a higher percent variation in female adults as compared to male adults. The stool samples that showed a higher variation had higher levels of lactoferrin prior to spiking. The results are illustrated in Tables 15 and 16 below.

Table 15: Stool samples of female adult subjects spiked to a final concentration of 25 ng/mL

Patient ID #	Actual Lactoferrin (ng/ML)	Estimated Lactoferrin (ng/mL)	Variation (%)
1	25	15.4	38.4
2	25	22.9	8.5
3	25	21.8	12.7
4	25	28.4	13.5
5	25	16.2	35.3
6	25	15.8	37.0
7	25	35.5	41.8
8	25	46.5	85.8
9	25	27.7	10.8
10	25	32.3	29.1

Patient ID #	Actual Lactoferrin (ng/ML)	Estimated Lactoferrin (ng/ml)	Variation (%)
11	25	26.1	4.3
12	25	25.3	1.0

Table 16: Stool samples of male adult subjects spiked to a final concentration of 25 ng/mL

Patient ID #	Actual Lactoferrin (ng/mL)	Estimated Lactoferrin (ng/mL)	Variation (%)
1	25	21.9	12.4
2	25	21.2	15.0
3	25	20.9	16.3
4	25	21.4	14.4
5	25	20.8	16.8
6	25	22.8	8.8
7	25	28.9	15.5
8	25	29.4	17.4
9	25	36.7	47.0
10	25	19.5	21.9

[0052] A second method for spiking was using the same two stool specimens collected six weeks apart from six female and five male adults were diluted and spiked with lactoferrin to a concentration of 4 μg/mL. The estimated lactoferrin represents the level of lactoferrin determined from a standard curve generated by the quantitative ELISA. The % Variation represents the difference between the actual amount used to spike the sample and the estimated value. Under these conditions, the variation ranged from 11.3% to 84.9% for females and from 5.0% to 39.2% for males. Results were similar to those obtained with specimens spiked with 25 ng/mL lactoferrin as described above, showing a higher percent variation in female adults compared to male adults. The results are illustrated in Tables 17 and 18 below.

Table 17: Stool samples of female adult subjects spiked to a final concentration of 4 µg/mL

Patient ID #	Actual Lactoferrin (µg/mL)	Estimated Lactoferrin (µg/mL)	Variation (%)
1	4	4.5	11.3
2	4	4.6	15.3
3	4	5.3	33.4
4	4	4.9	21.4
5	4	3.5	11.5
6	4	3.4	14.7
7	4	5.3	32.7
8	4	6.7	67.6
9	4	5.5	38.6
10	4	5.8	44.9
11	4	5.8	43.9
12	4	7.4	84.9

Table 18: Stool samples of male adult subjects spiked to a final concentration of 4 µg/mL

Patient ID #	Actual Lactoferrin (µg/mL)	Estimated Lactoferrin (µg/mL)	Variation (%)
1	4	4.7	17.5
2	4	4.6	14.4
3	4	4.2	5.0
4	4	5.6	39.2
5	4	4.2	5.9
6	4	4.7	18.5
7	4	4.7	16.5
8	4	5.5	37.9
9	4	5.3	33.6
10	4	4.3	6.6

Monitoring Using the Quantitative ELISA

[0053] The quantitative ELISA of the present invention was used to follow the lactoferrin levels of single patient suffering from ulcerative colitis during a flare of active disease through remission. The patient showed extremely high levels of lactoferrin (e.g.,

9749.37μg/mL feces) during the peak of the active disease, the levels dropping rapidly (e.g., to 7.42 μg/mL feces) following anti-inflammatory drug therapy. Levels elevated dramatically again during a relapse and leveled at slightly above those of healthy control persons (e.g., 11.06 μg/mL feces) during periods of remission. Thus, lactoferrin levels determined according to the quantitative ELISA of the present invention accurately depicted disease activity in response to medical treatment.

Example 3

ASCA Assay

[0054] In this example, a fecal sample was obtained and serially diluted 20 fold. 100 μl of the diluted sample was added to a test well of a microassay plate coated with extract of *Saccharomyces cerevisiae*. The sample then was incubated at 37°C to allow antibodies to *Saccharomyces cerevisiae* to bind to the extract of *Saccharomyces cerevisiae*. Following incubation, anti-human Ig polyclonal antibodies coupled to horseradish peroxidase enzyme (conjugate) were added to the test well and allowed to bind to captured ASCA. Unbound conjugate then was washed from the well and one component substrate (tetra-methyl-benzidene and hydrogen peroxide) was added for color development. Following the substrate incubation, 0.1M sulfuric acid was added to quench the reaction and the optical density (OD) was obtained spectrophotometrically at 450 nm using a single wavelength spectrophotometer.

[0055] The method described above was used in a clinical study to test a total of 86 IBD patients (55.8% males and 44.2% females). The approximate 1 to 1 ratio of males to females was similar to the ratio observed in IBD patient populations. The IBS patient group ranged in age from 19 to 78 years and was 9% male and 91% female. This

ratio of males to females (1:10) reflects the increased incidence for IBS in females as seen in patient populations. The healthy control (HC) patient group ranged in age from 20 to 79 years old and was 33.3% male and 66.6% female. A summary of the patient population in the clinical study is shown in Table 19.

Table 19: Summary of patient population.

Summary of Clinical Histories (N=120)	Total Subjects
Total number of IBD patients	86
No. Males	48
No. Females	38
Total number of patients with Crohn's Disease	49
No. Males	26
No. Females	23
Total number of patients with ulcerative colitis	37
No. Males	22
No. Females	15
Total number of patients with irritable bowel syndrome	22
No. Males	2
No. Females	20
Total number of healthy controls	12
No. Males	4
No. Females	8

[0056] In the clinical study, there were 37 ulcerative colitis patients, 49 Crohn's disease patients, 22 irritable bowel patients, and 12 healthy controls. Fecal samples were collected from each enrolled subject and stored at -70°C until tested. The optical densities for each sample were determined using the method described above. Results were reported as positive for fecal ASCA if an optical density of greater than or equal to 0.200 was observed. Results were reported as negative for fecal ASCA if an optical density of less than or equal to 0.199 was observed. Other clinical data, such as stool consistency, was also determined. Table 20, below, contains the clinical data and test

results for healthy patients that participated in this clinical study. Table 21, below, contains the clinical data and test results for patients with ulcerative colitis patients that participated in this clinical study. Table 22, below, contains the clinical data and test results for patients with Crohn's disease that participated in this study. Table 23, below, contains the clinical data and test results for patients with irritable bowel syndrome that participated in this study.

Table 20: Clinical data and test results for healthy controls

Donor ID	Sex	Age Range	Previous of chronic GI illness	Stool Consistency	Optical Density	Fecal ASCA
HC1	F	40 - 49	NO	Solid	0.098	NEGATIVE
HC2	F	40 - 49	NO	Solid	0.089	NEGATIVE
HC3	M	70 - 79	NO	Solid	0.095	NEGATIVE
HC4	F	60 - 69	NO	Solid	0.085	NEGATIVE
HC5	M	70 – 79	NO	Solid	0.083	NEGATIVE
HC6	F	70 – 79	NO	Solid	0.076	NEGATIVE
HC7	F	50 – 59	NO	Solid	0.124	NEGATIVE
HC8	F	40 – 49	NO	Solid	0.095	NEGATIVE
HC9	F	50 – 49	NO	Solid	0.111	NEGATIVE
HC10	F	40 – 49	NO	Solid	0.111	NEGATIVE
HC11	M	50 - 60	NO	Solid	0.070	NEGATIVE
HC12	M	50 - 60	NO	Solid	0.054	NEGATIVE

Table 21: Clinical data and test results for ulcerative colitis patients

Patient ID	Sex	Age	Disease	Stool Consistency	Disease Activity	Optical Density	Fecal ASCA
UC1	F	46	UC	Liquid	ACTIVE	0.184	NEGATIVE
UC2	M	39	UC	Liquid	ACTIVE	0.378	POSITIVE
UC3	F	30	UC	Semi-Solid	ACTIVE	0.193	NEGATIVE
UC4	F	31	UC	Semi-Solid	INACTIVE	0.319	POSITIVE
UC5	F	30	UC	Semi-Solid	ACTIVE	0.114	NEGATIVE
UC6	M	61	UC	Semi-Solid	INACTIVE	0.115	NEGATIVE
UC7	F	68	UC	Liquid	INACTIVE	0.091	NEGATIVE

UC8	F	45	UC	Liquid	ACTIVE	0.356	POSITIVE
UC9	F	21	UC	Semi-Solid	ACTIVE	0.082	NEGATIVE
UC10	F	27	UC	Liquid	ACTIVE	0.161	NEGATIVE
UC11	F	24	UC	Solid	INACTIVE	0.104	NEGATIVE
UC12	F	74	UC	Semi-Solid	INACTIVE	0.091	NEGATIVE
UC13	M	69	UC	Semi-Solid	ACTIVE	0.070	NEGATIVE
UC14	M	19	UC	Solid	INACTIVE	0.088	NEGATIVE
UC15	M	62	UC	Solid	INACTIVE	0.054	NEGATIVE
UC16	F	70	UC	Solid	INACTIVE	0.056	NEGATIVE
UC17	M	23	UC	Liquid	ACTIVE	0.573	POSITIVE
UC18	F	52	UC	Solid	ACTIVE	0.073	NEGATIVE
UC19	M	60	UC	Solid	INACTIVE	0.062	NEGATIVE
UC20	F	52	UC	Liquid	ACTIVE	0.089	NEGATIVE
UC21	M	31	UC	Solid	INACTIVE	0.064	NEGATIVE
UC22	M	44	UC	Semi-Solid	INACTIVE	0.143	NEGATIVE
UC23	F	30	UC	Liquid	ACTIVE	0.110	NEGATIVE
UC24	M	48	UC	Semi-Solid	INACTIVE	0.096	NEGATIVE
UC25	F	37	UC	Liquid	ACTIVE	0.282	POSITIVE
UC26	F	32	UC	Solid	ACTIVE	0.107	NEGATIVE
UC27	F	46	UC	Liquid	ACTIVE	0.199	NEGATIVE
UC28	M	49	UC	Semi-Solid	INACTIVE	0.161	NEGATIVE
UC29	F	42	UC	Solid	INACTIVE	0.080	NEGATIVE
UC30	F	41	UC	Semi-Solid	INACTIVE	0.087	NEGATIVE
UC31	F	43	UC	Solid	INACTIVE	0.070	NEGATIVE
UC32	M	30	UC	Solid	ACTIVE	0.103	NEGATIVE
UC33	F	43	UC	Solid	INACTIVE	0.092	NEGATIVE
UC34	F	33	UC	Semi-Solid	INACTIVE	0.075	NEGATIVE
UC35	M	58	UC	Semi-Solid	ACTIVE	0.121	NEGATIVE
UC36	F	32	UC	Semi-Solid	ACTIVE	0.083	NEGATIVE

Table 22: Clinical Data and test results for Crohn's disease patients.

Patient ID	Sex	Age	Disease	Stool Consistency	Disease Activity	Optical Density	FECAL ASCA
CD1	M	26	CD	Liquid	INACTIVE	1.900	POSITIVE
CD2	M	60	CD	Liquid	ACTIVE	2.849	POSITIVE
CD3	F	66	CD	Liquid	ACTIVE	0.282	POSITIVE

CD4	F	74	CD	Semi-Solid	INACTIVE	0.091	NEGATIVE
CD5	F	25	CD	Solid	INACTIVE	0.162	NEGATIVE
CD6	F	66	CD	Semi-Solid	INACTIVE	1.240	POSITIVE
CD7	M	39	CD	No Data	ACTIVE	1.150	POSITIVE
CD8	F	46	CD	Liquid	ACTIVE	0.160	NEGATIVE
CD9	F	46	CD	Semi-Solid	INACTIVE	0.074	NEGATIVE
CD10	F	56	CD	Solid	ACTIVE	0.406	POSITIVE
CD11	M	56	CD	Solid	ACTIVE	0.168	NEGATIVE
CD12	F	56	CD	Liquid	ACTIVE	0.732	POSITIVE
CD13	M	21	CD	Solid	ACTIVE	1.369	POSITIVE
CD14	M	52	CD	Semi-Solid	INACTIVE	0.136	NEGATIVE
CD15	M	63	CD	Solid	INACTIVE	0.134	NEGATIVE
CD16	M	34	CD	Solid	ACTIVE	0.076	NEGATIVE
CD17	F	45	CD	Semi-Solid	ACTIVE	0.160	NEGATIVE
CD18	M	67	CD	Semi-Solid	INACTIVE	0.059	NEGATIVE
CD19	F	46	CD	No Data	ACTIVE	0.839	POSITIVE
CD20	M	66	CD	Semi-Solid	INACTIVE	0.084	NEGATIVE
CD21	M	63	CD	Liquid	ACTIVE	0.780	POSITIVE
CD21	M	51	CD	Semi-Solid	ACTIVE	3.000	POSITIVE
CD22	M	34	CD	Semi-Solid	ACTIVE	1.447	POSITIVE
CD23	M	21	CD	Solid	ACTIVE	2.757	POSITIVE
CD24	F	78	CD	Semi-Solid	INACTIVE	0.092	NEGATIVE
CD25	F	27	CD	Semi-Solid	ACTIVE	0.979	POSITIVE
CD26	M	40	CD	Liquid	ACTIVE	0.373	POSITIVE
CD27	M	51	CD	Liquid	ACTIVE	0.978	POSITIVE
CD28	M	42	CD	Liquid	ACTIVE	0.089	NEGATIVE
CD29	F	31	CD	Solid	INACTIVE	0.075	NEGATIVE
CD30	F	59	CD	Solid	ACTIVE	0.088	NEGATIVE
CD31	M	35	CD	Semi-Solid	ACTIVE	1.487	POSITIVE
CD32	M	37	CD	Semi-Solid	INACTIVE	1.257	POSITIVE
CD33	F	77	CD	Solid	INACTIVE	0.093	NEGATIVE
CD34	F	40	CD	No Data	ACTIVE	1.762	POSITIVE
CD35	F	38	CD	Liquid	ACTIVE	0.098	NEGATIVE
CD36	M	51	CD	Liquid	ACTIVE	2.326	POSITIVE
CD37	M	38	CD	Semi-Solid	ACTIVE	0.091	NEGATIVE
CD38	M	37	CD	Liquid	ACTIVE	0.372	POSITIVE
CD39	M	59	CD	Semi-Solid	ACTIVE	0.224	POSITIVE
CD40	F	41	CD	Solid	ACTIVE	0.503	POSITIVE

CD41	M	41	CD	Solid	ACTIVE	0.117	NEGATIVE
CD42	M	48	CD	Liquid	ACTIVE	0.115	NEGATIVE
CD43	F	40	CD	Solid	INACTIVE	0.638	POSITIVE
CD44	F	72	CD	Solid	ACTIVE	0.087	NEGATIVE
CD45	F	32	CD	Liquid	INACTIVE	0.911	POSITIVE
CD46	F	24	CD	Liquid	ACTIVE	0.341	POSITIVE
CD47	M	23	CD	Solid	INACTIVE	0.088	NEGATIVE
CD48	F	34	CD	Liquid	ACTIVE	0.599	POSITIVE

Table 23: Clinical data and test results for irritable bowel syndrome patients

Patient ID	Sex	Age	Disease	Stool consistency	Disease Activity	Optical Density	Fecal ASCA
IBS1	F	56	IBS	Semi-Solid	ACTIVE	0.132	NEGATIVE
IBS2	F	48	IBS	Solid	ACTIVE	0.103	NEGATIVE
IBS3	F	30	IBS	Solid	ACTIVE	0.073	NEGATIVE
IBS4	F	31	IBS	Solid	ACTIVE	0.074	NEGATIVE
IBS5	F	72	IBS	Semi-Solid	ACTIVE	0.079	NEGATIVE
IBS6	F	47	IBS	Solid	ACTIVE	0.088	NEGATIVE
IBS7	F	19	IBS	Semi-Solid	ACTIVE	0.105	NEGATIVE
IBS8	F	58	IBS	Semi-Solid	ACTIVE	0.107	NEGATIVE
IBS9	F	40	IBS	Solid	ACTIVE	0.065	NEGATIVE
IBS10	F	33	IBS	Semi-Solid	ACTIVE	0.065	NEGATIVE
IBS11	F	78	IBS	Solid	ACTIVE	0.071	NEGATIVE
IBS12	F	74	IBS	Semi-Solid	ACTIVE	0.063	NEGATIVE
IBS13	F	50	IBS	Semi-Solid	ACTIVE	0.052	NEGATIVE
IBS14	F	39	IBS	Solid	ACTIVE	0.079	NEGATIVE
IBS15	F	54	IBS	Solid	ACTIVE	0.080	NEGATIVE
IBS16	M	49	IBS	Semi-Solid	ACTIVE	0.238	POSITIVE
IBS17	M	53	IBS	Solid	ACTIVE	0.123	NEGATIVE
IBS18	F	34	IBS	Solid	ACTIVE	0.091	NEGATIVE
IBS19	F	43	IBS	Solid	ACTIVE	0.075	NEGATIVE
IBS20	F	35	IBS	Solid	ACTIVE	0.075	NEGATIVE
IBS21	F	51	IBS	Semi-Solid	ACTIVE	0.081	NEGATIVE
IBS22	F	40	IBS	Solid	ACTIVE	0.083	NEGATIVE

[0057] There were a total of 49 patients with Crohn's disease and 37 with ulcerative colitis. In the Crohn's disease group, a total of 55.1% patients were positive for fecal ASCA. In the ulcerative colitis group, 13.5% were positive. Of the 22 IBS patients, a single patient (4.6%) was positive for fecal ASCA. All 12 healthy controls were negative. A summary of positive results for fecal ASCA is shown in Table 24.

Table 24: Summary of positive results for Crohn's disease, ulcerative colitis, active IBS, and healthy controls

Total Assessments N = 120	Total	Fecal ASCA Positive	Fecal ASCA Negative
Total IBD (Crohn's disease and ulcerative colitis)	**86**	**37.2% (32)**	**62.8% (54)**
Total Crohn's Disease	**49**	**55.1% (27)**	**44.9% (22)**
Total Ulcerative Colitis	**37**	**13.5% (5)**	**86.5% (32)**
Total Active IBS	**22**	**4.6% (1)**	**96.4% (21)**
Total Healthy Controls	**12**	**0**	**100.0% (12)**

[0058] When distinguishing Crohn's disease from ulcerative colitis, fecal ASCA exhibited a sensitivity of 55.1% and specificity of 86.5%. The predictive positive and negative values were 84.4% and 59.3%, respectively, and the correlation was 68.6% as shown in Table 25.

Table 25: Statistical evaluation using the presence of fecal ASCA to distinguish Crohn's disease from ulcerative colitis

N=86	Crohn's disease	Ulcerative colitis
Fecal ASCA positive	27	5
Fecal ASCA negative	22	32

Sensitivity	55.1%
Specificity	86.5%
Predictive Positive Value	84.4%
Predictive Negative Value	59.3%
Correlation	68.6%

[0059] When distinguishing Crohn's disease from ulcerative colitis, irritable bowel syndrome and healthy controls, fecal ASCA exhibited a sensitivity of 55% and a specificity of 91.6%. The predictive positive and negative values were 82% and 75%, respectively, and the correlation was 77% as shown below in Table 26.

Table 26: Statistical evaluation using fecal ASCA to distinguish Crohn's disease from ulcerative colitis, irritable bowel syndrome/healthy controls

N=120	Crohn's disease	UC/IBS/Healthy Controls
Fecal ASCA positive	27	6
Fecal ASCA negative	22	65

Sensitivity	55.1%
Specificity	91.6%
Predictive Positive Value	81.8%
Predictive Negative Value	74.7%
Correlation	76.7%

[0060] The mean optical densities for each group were obtained and differences were tested for statistical significance using a two-tailed t-test giving a p-value result. Of the 33 patients that tested positive for fecal ASCA, there were 27 CD, 5 UC, and 1 IBS.

Sensitivity, specificity and overall correlation were 55.1%, 91.5% and 76.7%, respectively. ASCA-positive CD showed a higher mean±SD A450 of 1.183±0.794 as compared to 0.382±0.113 for UC and the single A450 of 0.0.091±0.0.038 for IBS. There was a significant difference between CD and all other subject groups. A summary of the statistical analysis is listed in Table 27.

Table 27: Summary of the Mean and P values of Optical Densities for Fecal ASCA

Test Group	Mean Optical Density	Standard Deviation	Optical Density Range	P Value
CD	1.183	0.794	0.341-3.000	CD vs UC,IBS,HC $P < 0.005$
UC	0.382	0.113	0.382-0.113	CD vs UC $P < 0.05$
IBS	0.091	0.038	0.052-0.238	CD vs IBS $P < 0.005$
HC	0.091	0.019	0.054-0.124	CD vs HC $P < 0.005$

[0061] The sensitivity of the fecal ASCA test also was determined using serial two fold dilutions of highly purified ASCA antibodies. For the analysis, standard curves were generated using the kit diluent. The test was consistently positive at a concentration of 0.62 μg/mL as determined by a cutoff absorbency value of ≥ 0.200. Individual results are shown below in Table 28. The standard curves are shown in FIG. 1.

Table 28: Standard curves generated using purified ASCA antibodies

Purified ASCA Antibodies (µg/mL)	Test 1	Test 2	Mean	Std Dev
5.00	1.702	1.856	1.779	0.108
2.50	1.117	1.099	1.108	0.012
1.25	0.634	0.624	0.629	0.007
0.62	**0.303**	**0.329**	**0.316**	**0.018**
0.31	0.191	0.164	0.177	0.019
0.16	0.115	0.113	0.114	0.001
0.08	0.090	0.077	0.083	0.009
0.04	0.063	0.065	0.064	0.001

[0062] Tests also were conducted to determine what type of immunoglobulins (antibodies) were present in a fecal sample and in serum. The immunglobulin typing was done for human IgA, human $IgA_{sec,}$ human IgD, human IgM, and human IgG. The immunoglobulin typing was done on a fecal sample from 6 Crohn's disease patients and 2 ulcerative colitis and on a serum control sample using pre-absorbed Ig-type specific conjugates. The serum control sample was obtained from a patient with a confirmed allergy to *Saccharomyces cerevisiae*.

[0063] Of the Crohn's disease patients, 5 patients exhibited a response to IgA and $IgA_{sec,}$ 4 patients exhibited a response to IgM and a single patient exhibited a response to IgG. Of the 2 ulcerative colitis patients, a single patient reacted with the Ig conjugate. The serum control only exhibited a response to individual immunoglobulins IgM and IgG. A response to IgA and IgA_{sec} occurred the fecal samples but not in the control serum sample. A summary of results is shown in Table 29.

Table 29: A Summary of Immunoglobulin Typing of ASCA in a Human Fecal sample and a Serum Control

Patient Number	Disease	IgA Conjugate	IgA_{sec} Conjugate	IgD Conjugate	IgM Conjugate	IgG Conjugate	Ig Conjugate
1	Crohn's Disease	+	+	-	+	+	+
2	Crohn's Disease	+	+	-	+	-	+
3	Crohn's Disease	-	-	-	-	-	-
4	Crohn's Disease	+	+	NO DATA	+	-	+
5	Crohn's Disease	+	+	NO DATA	-	-	+
6	Crohn's Disease	+	+	NO DATA	+	-	+
7	Ulcerative Colitis	-	-	-	-	-	-
8	Ulcerative Colitis	-	-	-	-	-	+
Serum Control	Yeast Allergy	-	-	-	+	+	+

Example 4

ANCA ASSAY

[0064] The ANCA specific immunoassay was used to differentiate ulcerative colitis and other gastrointestinal illnesses such as Crohn's disease and irritable bowel

syndrome by measuring the level of total fecal ANCA. A qualitative immunoassay such as an enzyme-linked immunoassay that utilizes both monoclonal and polyclonal antibodies to endogenous human ANCA indicated the absence or presence of ulcerative colitis. In the example qualitative assay, the fecal specimen was diluted 10 fold and added to a well containing the immobilized neutrophil antigens. If ANCA was present, it was bound to the antigens during the incubation at 37°C. Following the incubation, anti-human Ig polyclonal antibodies coupled to horseradish peroxidase enzyme (conjugate) were added and allowed to bind to captured ANCA. Unbound conjugate was then washed from the well and one component substrate (tetramethybenzidene and hydrogen peroxide) was added for color development. Following the substrate incubation, 0.1M sulfuric acid was added to quench the reaction and the optical density (OD) was obtained spectrophotometrically at 450 nm.

[0065] Using the procedure described above, a total of 98 IBD patients were enrolled and comprised 51% males and 49% females with an age range of 0 to 69 years. The approximate 1 to 1 ratio is similar to the ratio observed in IBD patient populations. The IBS patient group had an age range of 5 to 39 years with 57% males and 43% females. The healthy controls were 55% male and 45% female and comprised the age range of 20 to 79 years. Individual numbers for each age group are shown in Table 30.

Table 30: Summary of patient population.

Summary of Clinical Histories (N=116)	Total Subjects
Total number of IBD patients	98
No. Males	50
No. Females	48

Total number of patients with Crohn's Disease	47
No. Males	26
No. Females	21
Total number of patients with ulcerative colitis	51
No. Males	24
No. Females	27
Total number of patients with irritable bowel syndrome	7
No. Males	4
No. Females	3
Total number of healthy persons	11
No. Males	6
No. Females	5

[0066] There were 51 ulcerative colitis patients, 47 Crohn's disease patients, 7 irritable bowel patients, and 11 healthy adults recruited for the study. Fecal specimens were collected from each enrolled patient and stored at -70°C until tested. Specimen consistency ranged from solid to liquid. The level of fecal ANCA was determined using the qualitative ANCA ELISA as previously described. Disease activity was defined using elevated fecal lactoferrin as an indicator of intestinal inflammation. A dilution of 1:10 was used in the *ANCA-CHEK* (qualitative ELISA) and results were reported as positive (absorbance values ≥ 0.140) or negative (absorbance values < 0.140). The mean optical densities, standard deviation and P values (two-tailed student T-test with unequal variance) were determined for the ANCA positive ulcerative colitis patients. Of the 26 patients that tested positive for fecal ANCA, there were 4 CD, 21 UC, and 1 healthy person. ANCA-positive UC showed a mean $\pm$ SD OD_{450} of 0.311 ± 0.166. The mean OD for the UC patients was significantly different from IBS and healthy persons (p value<0.0005). A summary of the statistical analysis is listed in Table 31.

Table 31: Summary of the mean, standard deviation and P values for *ANCA-CHEK* Optical densities

Group ID	Number	Mean Optical Density	Standard Deviation	Optical Density Range	P values
ANCA + UC	21	0.311	0.166	0.141-0.804	UC vs CD p<0.5
ANCA + CD	4	0.209	0.115	0.141-0.381	UC vs CD, IBS, H p<0.0005
IBS	7	0.078	0.027	0.047-0.121	UC vs CD, IBS p<0.005
Healthy	11	0.071	0.041	0.039-0.104	UC vs IBS, H p<0.0005

[0067] In the IBD group, there were 47 with Crohn's disease and 51 with ulcerative colitis. In the ulcerative colitis group, 41% were positive. In the Crohn's disease group, a total of 9% patients were positive by the *ANCA-CHEK*. Of the 11 healthy persons, 1 was positive and all 7 IBS patients were negative by the *ANCA-CHEK* test. A summary of positive results for the *ANCA-CHEK* is shown in Table 32 and individual results are listed in Tables 33 through 34.

Table 32: Summary of positive results for Crohn's disease, ulcerative colitis, and IBS

Total Assessments N = 116	Total	Fecal ANCA Positive	Fecal ANCA Negative
Total IBD (Crohn's disease and ulcerative colitis)	98	26% (25)	75% (73)
Total Crohn's Disease	47	9% (4)	91% (43)
Total Ulcerative Colitis	51	41% (21)	59% (30)
Total IBS	7	0	7
Total Healthy Persons	11	9%(1)	91%(10)

[0068] When distinguishing ulcerative colitis from Crohn's disease, the *ANCA-CHEK* exhibited a sensitivity of 41% and specificity of 92%. The predictive positive and negative values were 84% and 59%, respectively, and the correlation was 65% (Table 33).

Table 33: Statistical evaluation using the *ANCA-CHEK* to distinguish Crohn's disease from ulcerative colitis

N=98	Ulcerative colitis	Crohn's disease
ANCA-CHEK positive	21	4
ANCA-CHEK negative	30	43

Sensitivity	41%
Specificity	92%
Predictive Positive Value	84%
Predictive Negative Value	59%
Correlation	65%

[0069] When distinguishing ulcerative colitis from irritable bowel syndrome and healthy persons, the *ANCA-CHEK* exhibited a sensitivity of 41% and a specificity of 92%. The predictive positive and negative values were 81% and 67%, respectively, and the correlation was 70% (Table 34).

Table 34: Statistical evaluation using the *ANCA-CHEK* to distinguish ulcerative colitis from Crohn's disease, irritable bowel syndrome and healthy persons

N=116	Ulcerative colitis	Crohn's disease IBS/Healthy
ANCA-CHEK positive	21	5
ANCA-CHEK negative	30	60

Sensitivity	41%
Specificity	92%
Predictive Positive Value	81%
Predictive Negative Value	67%
Correlation	70%

[0070] The sensitivity of the *ANCA-CHEK* was determined using serial two fold dilutions of human ANCA positive serum. For the analysis, standard curves were generated using the sample diluent. The test was consistently positive to a titer of 0.063 as determined by a cutoff absorbance value of $\geq$ 0.200. Individual results are shown below in Table 35 and standard curves are shown in FIG. 2.

Table 35: Standard curves generated using *ANCA-CHEK* (cut-offs are bolded)

Human ANCA Serum	Test 1	Test 2	Test 3	Mean	Std Dev
1.000 (Neat)	1.441	1.469	1.525	1.478	0.043
0.500	1.098	0.941	1.014	1.018	0.079
0.250	0.717	0.595	0.666	0.659	0.061
0.125	0.492	0.428	0.444	0.455	0.033
0.063	**0.327**	0.303	0.320	0.317	0.012
0.032	0.196	**0.295**	**0.221**	**0.237**	0.051
0.016	0.132	0.184	0.179	0.165	0.029
Diluent	0.067	0.093	0.109	0.090	0.021

[0071] Table 36, below contains the clinical data and test results for patients with ulcerative colitis that participated in the study. Table 37, below, contains the clinical data and test results for patients with Crohn's disease that participated in the study. Table 38, below, contains the clinical data and test results for patients with irritable bowel syndrome that participated in the study. Table 39, below, contains the clinical data and test results for healthy patients that participated in the study.

Table 36: Clinical and ELISA results for ulcerative colitis patients.

Patient ID	Sex	Age Range	Disease	Disease Activity	*ANCA-CHEK* OD_{450}	*ANCA-CHEK* Result
UC1	F	10-19	UC	INACTIVE	0.053	NEGATIVE
UC2	F	5-9	UC	INACTIVE	0.107	NEGATIVE
UC3	F	5-9	UC	ACTIVE	0.058	NEGATIVE
UC4	M	10-19	UC	INACTIVE	0.048	NEGATIVE
UC5	M	10-19	UC	ACTIVE	0.512	POSITIVE
UC6	F	10-19	UC	ACTIVE	0.061	NEGATIVE
UC7	M	5-9	UC	ACTIVE	0.211	POSITIVE
UC8	M	10-19	UC	ACTIVE	0.106	NEGATIVE
UC9	M	10-19	UC	INACTIVE	0.804	POSITIVE
UC10	M	10-19	UC	ACTIVE	0.091	NEGATIVE
UC11	F	10-19	UC	ACTIVE	0.169	POSITIVE
UC12	F	10-19	UC	ACTIVE	0.209	POSITIVE
UC13	F	10-19	UC	ACTIVE	0.351	POSITIVE
UC14	F	10-19	UC	ACTIVE	0.198	POSITIVE
UC15	F	5-9	UC	ACTIVE	0.098	NEGATIVE
UC16	F	5-9	UC	ACTIVE	0.050	NEGATIVE
UC17	F	10-19	UC	ACTIVE	0.091	NEGATIVE
UC18	M	10-19	UC	ACTIVE	0.603	POSITIVE
UC19	M	10-19	UC	ACTIVE	0.091	NEGATIVE
UC20	F	10-19	UC	ACTIVE	0.142	POSITIVE
UC21	M	10-19	UC	ACTIVE	0.074	NEGATIVE
UC22	F	10-19	UC	ACTIVE	0.105	NEGATIVE
UC23	M	10-19	UC	INACTIVE	0.256	POSITIVE
UC24	F	0-4	UC	ACTIVE	0.308	POSITIVE
UC25	F	5-9	UC	ACTIVE	0.072	NEGATIVE
UC26	M	10-19	UC	INACTIVE	0.237	POSITIVE
UC27	M	10-19	UC	ACTIVE	0.048	NEGATIVE
UC28	M	10-19	UC	ACTIVE	0.049	NEGATIVE
UC29	M	10-19	UC	ACTIVE	0.059	NEGATIVE
UC30	F	10-19	UC	INACTIVE	0.047	NEGATIVE
UC31	M	10-19	UC	ACTIVE	0.055	NEGATIVE
UC32	M	10-19	UC	INACTIVE	0.044	NEGATIVE
UC33	F	10-19	UC	ACTIVE	0.043	NEGATIVE

UC34	M	5-9	UC	ACTIVE	0.046	NEGATIVE
UC35	M	10-18	UC	INACTIVE	0.043	NEGATIVE
UC36	M	10-17	UC	INACTIVE	0.040	NEGATIVE
UC37	F	10-19	UC	ACTIVE	0.047	NEGATIVE
UC38	F	0-4	UC	ACTIVE	0.049	NEGATIVE
UC39	F	5-9	UC	INACTIVE	0.363	POSITIVE
UC40	F	10-19	UC	INACTIVE	0.046	NEGATIVE
UC41	M	10-19	UC	ACTIVE	0.118	NEGATIVE
UC42	F	50-59	UC	ACTIVE	0.230	POSITIVE
UC43	M	10-19	UC	ACTIVE	0.051	NEGATIVE
UC44	F	30-39	UC	ACTIVE	0.060	NEGATIVE
UC45	F	50-59	UC	ACTIVE	0.465	POSITIVE
UC46	M	50-59	UC	ACTIVE	0.274	POSITIVE
UC47	F	30-39	UC	ACTIVE	0.141	POSITIVE
UC48	M	60-69	UC	ACTIVE	0.184	POSITIVE
UC49	F	40-49	UC	ACTIVE	0.397	POSITIVE
UC50	F	40-49	UC	ACTIVE	0.337	POSITIVE
UC51	M	30-39	UC	ACTIVE	0.143	POSITIVE

Table 37: Clinical and ELISA results for Crohn's disease patients.

Patient ID	Sex	Age Range	Disease	Disease Activity	*ANCA-CHEK* OD_{450}	*ANCA-CHEK* Result
CD1	M	10-19	CD	ACTIVE	0.050	NEGATIVE
CD2	M	10-19	CD	ACTIVE	0.113	NEGATIVE
CD3	M	10-19	CD	ACTIVE	0.050	NEGATIVE
CD4	F	10-19	CD	ACTIVE	0.381	POSITIVE
CD5	F	10-19	CD	ACTIVE	0.058	NEGATIVE
CD6	M	10-19	CD	INACTIVE	0.068	NEGATIVE
CD7	M	10-19	CD	ACTIVE	0.066	NEGATIVE
CD8	M	5-9	CD	ACTIVE	0.059	NEGATIVE
CD9	F	10-19	CD	ACTIVE	0.059	NEGATIVE
CD10	F	10-19	CD	ACTIVE	0.065	NEGATIVE
CD11	F	10-19	CD	INACTIVE	0.055	NEGATIVE
CD12	M	10-19	CD	INACTIVE	0.071	NEGATIVE
CD13	F	10-19	CD	ACTIVE	0.065	NEGATIVE

CD14	M	10-19	CD	ACTIVE	0.098	NEGATIVE
CD15	F	10-19	CD	ACTIVE	0.099	NEGATIVE
CD16	M	10-19	CD	ACTIVE	0.166	POSITIVE
CD17	F	10-19	CD	ACTIVE	0.147	POSITIVE
CD18	M	10-19	CD	ACTIVE	0.057	NEGATIVE
CD19	F	10-19	CD	ACTIVE	0.084	NEGATIVE
CD20	M	10-19	CD	ACTIVE	0.053	NEGATIVE
CD21	F	10-19	CD	ACTIVE	0.074	NEGATIVE
CD22	M	10-19	CD	ACTIVE	0.054	NEGATIVE
CD23	M	0-5	CD	ACTIVE	0.055	NEGATIVE
CD24	M	10-19	CD	ACTIVE	0.067	NEGATIVE
CD25	M	10-19	CD	ACTIVE	0.099	NEGATIVE
CD26	M	5-9	CD	ACTIVE	0.086	NEGATIVE
CD27	F	10-19	CD	ACTIVE	0.043	NEGATIVE
CD28	F	10-19	CD	ACTIVE	0.064	NEGATIVE
CD29	M	5-9	CD	INACTIVE	0.039	NEGATIVE
CD30	M	10-19	CD	ACTIVE	0.071	NEGATIVE
CD31	F	10-15	CD	ACTIVE	0.109	NEGATIVE
CD32	M	10-19	CD	INACTIVE	0.057	NEGATIVE
CD33	M	10-19	CD	ACTIVE	0.141	POSITIVE
CD34	M	10-19	CD	INACTIVE	0.045	NEGATIVE
CD35	F	10-19	CD	ACTIVE	0.051	NEGATIVE
CD36	F	10-19	CD	ACTIVE	0.132	NEGATIVE
CD37	F	10-19	CD	INACTIVE	0.046	NEGATIVE
CD38	M	10-19	CD	ACTIVE	0.057	NEGATIVE
CD39	F	20-29	CD	INACTIVE	0.051	NEGATIVE
CD40	F	20-29	CD	ACTIVE	0.053	NEGATIVE
CD41	M	50-59	CD	ACTIVE	0.060	NEGATIVE
CD42	F	50-59	CD	ACTIVE	0.062	NEGATIVE
CD43	M	20-29	CD	ACTIVE	0.056	NEGATIVE
CD44	F	60-69	CD	ACTIVE	0.130	NEGATIVE
CD45	M	60-69	CD	ACTIVE	0.078	NEGATIVE
CD46	F	40-49	CD	ACTIVE	0.116	NEGATIVE
CD47	M	60-69	CD	ACTIVE	0.057	NEGATIVE

Table 38: Clinical and ELISA results for Irritable bowel syndrome patients.

Patient ID	Sex	Age Range	Disease	*ANCA-CHEK* OD_{450}	*ANCA-CHEK* Results
IBS1	F	10-19	IBS	0.056	NEGATIVE
IBS2	M	10-19	IBS	0.047	NEGATIVE
IBS3	M	5-9	IBS	0.099	NEGATIVE
IBS4	M	10-19	IBS	0.068	NEGATIVE
IBS5	M	10-19	IBS	0.092	NEGATIVE
IBS6	F	20-29	IBS	0.121	NEGATIVE
IBS7	F	30-39	IBS	0.064	NEGATIVE

Table 39: Clinical and ELISA results for healthy persons.

Subject ID	Sex	Age Range	*ANCA-CHEK* OD_{450}	*ANCA-CHEK* Results
D1	F	40-49	0.087	NEGATIVE
D2	M	20-29	0.078	NEGATIVE
D5	M	20-29	0.178	POSITIVE
D15	M	50-59	0.041	NEGATIVE
D17	M	50-59	0.039	NEGATIVE
D18	F	40-49	0.069	NEGATIVE
D19	F	60-69	0.050	NEGATIVE
D20	M	70-79	0.039	NEGATIVE
D21	F	70-79	0.104	NEGATIVE
D22	M	60-69	0.045	NEGATIVE
D24	F	50-59	0.054	NEGATIVE

[0072] In summary, the present invention is directed to a method for the differentiation of inflammatory bowel disease (IBD) from irritable bowel disease (IBS) followed by distinguishing ulcerative colitis and Crohn's disease from other gastrointestinal illnesses. This highly differential method first uses the presence of elevated fecal lactoferrin as a marker of intestinal inflammation to differentiate IBD from

IBS. Patients suspected of IBD are then analyzed for fecal anti-*Saccharomyces cerevisiae* antibodies (ASCA) as an indicator of Crohn's disease and fecal anti-neutrophil cytoplasmic antibodies (ANCA) as an indicator of ulcerative colitis. IBD patients are further monitored for intestinal inflammation using fecal lactoferrin to evaluate the effectiveness of medical therapy and predict relapse. The apparatus consists of either a qualitative enzyme-linked immunoassay or other immunoassay that utilizes antibodies specific to total endogenous lactoferrin, ASCA and ANCA in human feces.

[0073] The method and apparatus may be used by healthcare providers to identify IBD and distinguish ulcerative colitis and Crohn's disease from other gastrointestinal illnesses. The present invention has been described in relation to particular embodiments, which are intended in all respects to be illustrative rather than restrictive. Alternative embodiments will become apparent to those skilled in the art to which the present invention pertains without departing from its scope.

[0074] From the foregoing, it will be seen that this invention is one well adapted to attain all the ends and objects hereinabove set forth together with other advantages which are obvious and which are inherent to the method.

[0075] It will be understood that certain features and subcombinations are of utility and may be employed without reference to other features and subcombinations. This is contemplated by and is within the scope of the claims.